

# Multiplicity Reactivation of Vaccinia Virus Particles Treated with Nitrogen Mustard

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The nitrogen mustard bis( $\beta$ -chloroethyl)methylamine, a radiomimetic alkylating agent, is known for its ability to inactivate viruses in general and poxvirus in particular. These experiments show that vaccinia virus is rapidly inactivated by it. They show, in addition, that the treated virus exhibits multiplicity reactivation in L cells, closely resembling that experienced with the same virus after exposure to ultraviolet rays. These findings have come from observations of plaque titer, on the one hand, and virus particle aggregation (measured in the electron microscope), on the other. The titer of the treated virus is very sensitive to particle aggregation, increasing and decreasing in a reversible manner as the degree of clumping among the particles is changed. The magnitude of these changes is such that they must be considered in any situation involving plaque assay of the fraction of virus surviving treatment with nitrogen mustard.

Some of the bacteriophages, after exposure to 2,537-A ultraviolet (UV) rays, produce more plaques in high multiplicity encounters with host cells than they do when the cells receive only one phage particle each. This is multiplicity reactivation (MR), first observed and named by Luria (6) in 1947. This phenomenon was detected with poxvirus by Abel (1) and measured in considerable detail in this laboratory (2, 9). We have learned that the ever-present aggregation that exists among the particles of poxvirus suspensions, although invalidating the usual estimates of multicomplex frequency via the Poisson function, can be turned to good advantage in experiments for demonstration and measurement of MR.

At high input multiplicity of the usual aggregated poxvirus preparation, the number of cells receiving two or more virus particles (VP) will consist, in part, of those receiving two or more separate VP by coincidence encounter and, in part, of cells that receive an aggregate of two or more VP. The combined effect of these two sources of multiple infection is exceedingly difficult to evaluate, but the effect of VP aggregation alone is measurable. Our procedure consists of observing and measuring the degree of VP aggregation in an electron microscope, then applying the same suspension to the cell monolayer in sufficiently low concentration that coincident encounters of two or more separate

VP with a cell are rare. Only multiplexes arising from encounters of cells with VP aggregates are significant, and the number of these can be predicted directly by electron microscopy.

Substantial MR has been reported only for viruses that have been exposed to radiation damage. Heated or urea-treated virus, for example, does not exhibit this effect. Some of the radiomimetic alkylating agents, notably the nitrogen mustard bis( $\beta$ -chloroethyl)methylamine, have been tested by Loveless and Stock (5) for their effects on T2 bacteriophage of *Escherichia coli*. A "very low degree" of MR was observed. We have thought the T2 phage to be a rather special case involving, as it does, a mode of entry to the cell which is entirely different from that of animal viruses. We therefore investigated the behavior of vaccinia virus after exposure to this nitrogen mustard and encountered MR as strong as that with UV-irradiated virus.

## MATERIALS AND METHODS

The WR (mouse neurotropic) strain of vaccinia virus was used in this work. Its culture in L cells and the details of plaque titration of this virus in monolayer bottle cultures of L cells have been adequately described elsewhere (9). Treatment of the virus with nitrogen mustard was the same as described by Joklik (3). Mixtures of virus containing 100  $\mu$ g (per ml) of freshly prepared bis( $\beta$ -chloroethyl)methylamine were incubated for 15 min at 37 C, and then were added to equal volumes of 10% sodium thiosulfate. This

TABLE 1. *Typical changes wrought in a vaccinia virus suspension by short exposure to 20-kc ultrasonic waves after treatment of the virus with nitrogen mustard<sup>a</sup>*

Treatment time	VP/ml	Single particles	PFU/ml	VP/PFU	AU/ml	AU/PFU (1/q)	q × 10 <sup>4</sup>	Slope of log-log line
sec		%						
0	4.2 × 10 <sup>7</sup>	13	6.8 × 10 <sup>5</sup>	62	1.7 × 10 <sup>7</sup>	2.5 × 10 <sup>1</sup>	400	1.69
10	4.3 × 10 <sup>7</sup>	57	1.0 × 10 <sup>4</sup>	4,300	3.1 × 10 <sup>7</sup>	3.1 × 10 <sup>3</sup>	3.2	2.82
60	4.8 × 10 <sup>7</sup>	82	4.5 × 10 <sup>2</sup>	107,000	4.1 × 10 <sup>7</sup>	9.1 × 10 <sup>4</sup>	0.11	4.32

<sup>a</sup> Prior to mustard treatment, this virus produced 1,030 PFU per 10,000 AU. Symbols: VP = virus particles; PFU = plaque-forming units; AU = suspended or active unit of virus (single particle or an aggregate); q = virus quality. Slope of log-log line is calculated in the units of Fig. 1.

mixture was incubated for an additional 2 hr at room temperature to neutralize the residual reagent.

VP number and degree of aggregation were determined by electron microscopy. Dilute suspensions of the VP were sedimented by centrifuge upon a smooth agar surface and examined by pseudoreplica by the method of Sharp (8). Counts were made of the number of single particles, pairs, triplets, etc., and these were used to determine the overall VP content of each inoculum titrated, as well as its content of aggregates in each size category.

The degree of VP aggregation present in the various inocula was controlled in one of two ways. The dispersion of aggregated suspensions was increased by application of 20,000 cycles/sec of vibrational energy from the microtip of a model 75 Branson Sonifier (4). Increases in aggregation of the VP in dispersed preparations of high concentration (10<sup>9</sup> or more VP per milliliter) occurred spontaneously on standing. With dispersed preparations of lower concentration, increases in aggregation were achieved by sedimenting the dispersed virus in a round-bottomed test tube in a horizontal centrifuge operating for 30 min at 10,000 × g (at the pellet) and 10 C. After resuspension of the virus in the same fluid by vigorous pipette action, the percentage of single VP had usually been reduced to less than 20. Such preparations are denoted as "aggregated virus."

## RESULTS

Fresh virus (161st L cell passage) was released from infected L cells by treatment for 1 min with ultrasonic (20 kc) waves. Sufficient phosphate-buffered saline (PBS), at pH 7.2, and 0.1% nitrogen mustard were added to make a mixture containing 100 μg of the reagent and 2.79 × 10<sup>9</sup> VP per ml. After treatment and immediate neutralization of excess nitrogen mustard by the addition of sodium thiosulfate, the virus was frozen at -20 C for titration and aggregation analysis the next day, or later.

After thawing and appropriate dilution of a part of the treated virus with PBS, electron micrographs were made for VP count and aggregation analysis, and monolayer bottles of L cells were inoculated for plaque titration. The results (top line of Table 1) reveal a high degree of VP

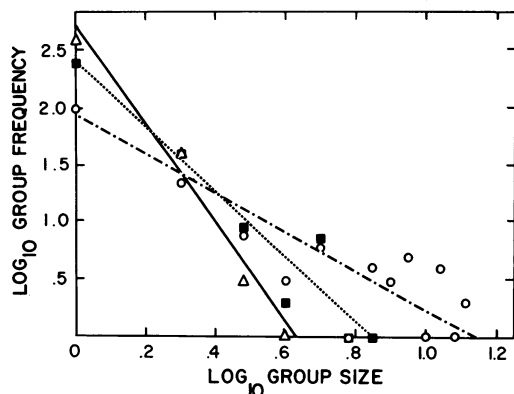


FIG. 1. *Distribution of aggregated group frequency as a function of group size. These three log-log lines represent the three degrees of virus particle dispersion observed in the experiment of Table 1. The lines of least, middle, and greatest slope (last column of Table 1) correspond to 13, 57, and 82% single particles, respectively.*

aggregation (13% single particles) as well as a high degree of survival of plaque-forming power. The virus produced 1,030 plaques per 10,000 suspended or active units (AU) prior to treatment with nitrogen mustard. This number had declined only to 400; however, when another part of the treated virus was exposed to 10 sec of vibrations at 20 kc and the fraction of single particles was thereby increased to 57% (second line of Table 1), plaque production decreased to 3.2 per 10<sup>4</sup> AU. After 60 sec at 20 kc, only 18% of the VP remained in the aggregated state, and the plaque production was 0.11 plaque-forming units (PFU) per 10,000 AU. No significant change occurred in the total count of VP nor in their appearance in the electron micrographs; only the degree of aggregation was measurably altered by the 20-kc waves.

The state of particle aggregation is apparently critical. The single statistic, "per cent of single particles," is inadequate to describe the distribution of group sizes, so graphs were prepared

TABLE 2. Decline in plaquing efficiency during extended treatment of vaccinia virus with 20-kc ultrasonic waves after exposure to nitrogen mustard<sup>a</sup>

Treatment time	VP/ml	Single particles	PFU/ml	VP/PFU	AU/ml	AU/PFU (1/q)	q × 10 <sup>5</sup>
min		%					
0	1.02 × 10 <sup>7</sup>	18	4.87 × 10 <sup>4</sup>	2.1 × 10 <sup>2</sup>	2.9 × 10 <sup>6</sup>	6.0 × 10 <sup>1</sup>	1,667
1	0.89 × 10 <sup>7</sup>	76	2.07 × 10 <sup>3</sup>	4.3 × 10 <sup>3</sup>	7.7 × 10 <sup>6</sup>	3.72 × 10 <sup>3</sup>	27
2	1.18 × 10 <sup>7</sup>	54	1.65 × 10 <sup>3</sup>	7.2 × 10 <sup>3</sup>	8.3 × 10 <sup>6</sup>	5.0 × 10 <sup>3</sup>	20
3	1.06 × 10 <sup>7</sup>	61	8.2 × 10 <sup>2</sup>	1.3 × 10 <sup>4</sup>	8.1 × 10 <sup>6</sup>	9.9 × 10 <sup>3</sup>	10
4	0.8 × 10 <sup>7</sup>	62	3.9 × 10 <sup>2</sup>	2.1 × 10 <sup>4</sup>	6.2 × 10 <sup>6</sup>	1.6 × 10 <sup>4</sup>	6

<sup>a</sup> Five portions of the virus preparation were treated for different lengths of time. In the first 1 min, there was a 62-fold reduction in PFU per AU (last column) as well as a major change in particle dispersion. Subsequent changes were small.

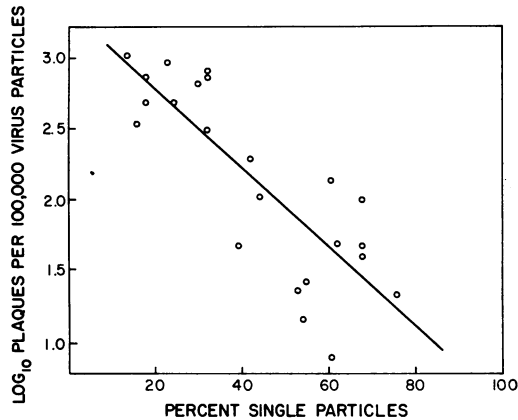


FIG. 2. Inactivation of single virus particles by exposure to nitrogen mustard. Surviving plaque titer, shown here, arises principally from aggregates. Changes in dispersion of this treated virus may change plaque titer 100-fold.

showing the frequency of each group size in the three samples of Table 1. These were plotted in log-log form (Fig. 1), because the aggregation of fresh virus gives a straight line when plotted in this way (4). Mustard-treated virus too is very nearly linear in this respect, so we have drawn straight lines and used their slopes, in the last column of Table 1, as a second statistic descriptive of the physical state of the virus suspension.

The effect of protracted 20-kc vibrations on nitrogen mustard-treated virus was shown in other experiments. The results of one of these are shown in Table 2, in which the plaquing efficiency of the virus was reduced by a factor of 62 in the sample treated for 1 min. Further treatment of the virus revealed a continuing but much slower rate of decline (4.5 × between samples treated 1 and 4 min, respectively).

Several other types of experiments were done, to learn whether 20-kc mechanical vibrations reduced virus titer by separating particle aggre-

gates or by destroying the particles or perhaps by both. The amount of aggregation remaining in a suspension of particles is not determined entirely by the amount of 20-kc treatment it has sustained. Treatment at high or low particle concentration, for example, or subsequent sedimentation and resuspension in a centrifuge, can yield suspensions of various degrees of aggregation, all of which may have received the same amount of 20-kc treatment. Physical data and plaque titers were collected from a series of experiments with virus, none of which received over 2 min of 20-kc treatment after nitrogen mustard treatment. These were plotted (Fig. 2) as the PFU-VP ratio against the state of dispersion expressed as the percentage of single particles found in the electron microscope analyses. Figure 2 shows that the titer of a given number of these nitrogen mustard-treated VP is very much greater when they are aggregated.

Reversibility of titer change was examined by putting the mustard-treated virus through several cycles of deaggregation by 20-kc treatment and reaggregation in the centrifuge. Detailed data from two such experiments are shown in Table 3. In these experiments there was usually some loss of virus during the sedimentation and resuspension operations, so it is not the PFU per milliliter but rather the ratios of PFU to VP and to AU that are compared with the degree of aggregation in the column showing the percentage of single particles. These ratios all show a high degree of reversibility in spite of the fact that the virus was dispersed each time by an additional treatment for 60 sec with 20-kc waves.

Finally, a single experiment was performed which shows the remarkably close correlation between plaque titer of mustard-treated virus and the state of aggregation of its particles. This experiment (Fig. 3) shows the correlation of these two variables not only during the course of a short (30 sec) application of 20-kc waves, but

TABLE 3. *Loss in plaque titer of mustard-treated vaccinia virus on exposure to 20-kc ultrasonic waves, and reversal<sup>a</sup> when the dispersed virus is reaggregated*

Expt	Sample	VP/ml	PFU/ml	AU/ml	Single particles	VP/PFU	AU/PFU (1/q)	q × 10 <sup>4</sup>	Change in q	
1	Control <sup>b</sup>	7.32 × 10 <sup>6</sup>	2.57 × 10 <sup>5</sup>	2.49 × 10 <sup>6</sup>	%					
	Aggregated	6.34 × 10 <sup>6</sup>	6.7 × 10 <sup>4</sup>	1.39 × 10 <sup>6</sup>	21	28	9.7	1,030		
	Dispersed	4.54 × 10 <sup>6</sup>	4.4 × 10 <sup>3</sup>	3.72 × 10 <sup>6</sup>	14	95	21	476		
	Reaggregated	9.49 × 10 <sup>6</sup>	3.21 × 10 <sup>4</sup>	2.17 × 10 <sup>6</sup>	68	1,030	845	12	40	× drop
2	Aggregated	32.3 × 10 <sup>6</sup>	2.34 × 10 <sup>5</sup>	9.21 × 10 <sup>6</sup>	18	138	39	256		
	Dispersed	9.9 × 10 <sup>6</sup>	1.37 × 10 <sup>4</sup>	7.39 × 10 <sup>6</sup>	61	723	539	19	13	× drop
	Reaggregated	11.3 × 10 <sup>6</sup>	3.43 × 10 <sup>4</sup>	5.36 × 10 <sup>6</sup>	32	329	156	64	3.4	× rise
	Redispersed	7.3 × 10 <sup>6</sup>	2.8 × 10 <sup>3</sup>	5.94 × 10 <sup>6</sup>	68	2,610	2,120	5	13	× drop
	Reaggregated again	8.4 × 10 <sup>6</sup>	7.9 × 10 <sup>4</sup>	2.78 × 10 <sup>6</sup>	23	106	35	286	57	× rise

<sup>a</sup> This reversal process can be repeated. Compare single particles with virus quality, q.

<sup>b</sup> Not treated with nitrogen mustard.

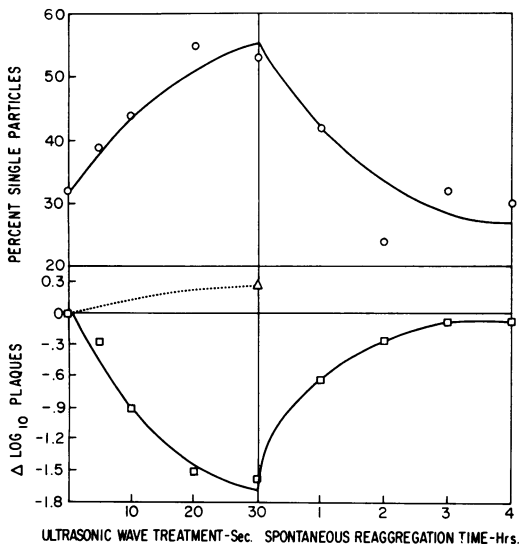


FIG. 3. *Dependence (reversible) of plaque titer on particle aggregation of vaccinia virus after treatment with nitrogen mustard. Upper curve shows dispersion of virus by ultrasonic waves followed by spontaneous reaggregation on standing. Lower curve shows fall and subsequent return of titer. Dotted line represents control virus that received neutralizing sodium thiosulfate but no nitrogen mustard.*

also during the succeeding 4 hr during which the virus suspension, which was initially quite concentrated, reaggregated spontaneously to its original condition. The plaque titer, that had declined to less than one-sixtieth of its original value during the dispersal of the virus, returned to its original high level as the aggregates reformed. The behavior of virus treated only with

sodium thiosulfate (used in all nitrogen mustard experiments to neutralize the reagent after the desired treatment) is shown also in Fig. 3. The dotted line indicates that this virus not only does not decrease in titer when dispersed by 20-kc waves, but also, like fresh virus, it increases somewhat as infectious individual VP are released from aggregates and thus are able to act freely as individuals.

#### DISCUSSION

Partly because of the low plaquing efficiency (7), but mainly because of a tendency for vaccinia virus to form stable aggregates in suspension, it is exceedingly difficult if not impossible to make any quantitative measurements of MR of this virus by employing the methods of those who have worked with the well-dispersed coliphages. The effects of multiple infection, or, more correctly, infection resulting from the combined effects of two or more nitrogen mustard-treated virus particles, can, however, be clearly seen in such experiments as these, in which the state of aggregation is changed in relatively dilute inocula. In these experiments, the behavior of mustard-treated virus stands in sharp contrast to that of fresh or heated virus, both of which increase in plaque titer when aggregates of particles are dispersed. The decrease in titer seen with mustard-treated virus is very similar, in fact, to that of UV-irradiated virus (9). The similarity is more than superficial; it extends to the actual magnitude of the reactivation involved. A comparison is possible if we examine two samples of the same well-dispersed vaccinia virus, reduced in plaque titer to approximately the same extent, the one by exposure to UV rays

and the other by nitrogen mustard. If the plaque titers of these two samples of damaged virus are similarly sensitive to changes in particle aggregation, we may safely say that they are similar in the magnitude of their MR. A comparison of Table 3 of this paper with Table 1 of our previous publication (9) shows that this is indeed true.

Joklik (3) showed that mustard-inactivated virus is still able to act as "uncoating" agent for other virus (heated) that is genetically intact but incapable, itself, of providing the uncoating stimulus. He therefore concluded that a treatment with nitrogen mustard that is capable of destroying the infectivity of the VP may leave the protein of the poxvirus essentially intact. His work showed also that the deoxyribonucleic acid (DNA) of UV-irradiated virus is not uncoated within the cell. Thus, there is a major difference in the competence of the protein of these two altered viruses. Our work now shows both to be about equally responsive in MR in the L cell. This leaves unanswered the question of uncoating of UV-treated virus during MR but suggests that both nitrogen mustard and UV photons produce random damage in some essential structure, probably the DNA. Infection of the cell occurs in the presence of two or more, but not one, of these particles. This would seem to be the result of a combination of randomly damaged parts rather than augmentation by several similarly damaged parts.

Previous attempts to produce MR among bacteriophages treated with alkylating agents have been generally unsuccessful. To our knowledge, only the report of Loveless and Stock (5) indicates positive results, and that only to a "low degree" compared with that observed after UV irradiation of the phage. It is possible that the inactivation of the phages, particularly T2 treated with nitrogen mustard, was partly in the form of damage to the injection mechanism. Infection of cells by such similarly damaged VP might possibly be augmented at high multiplicity (weak MR), but there would be no possibility for

recombination of differently (randomly) damaged particles and consequently no strong MR.

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